TRANSGENIC PLANTS WITH TOCOPHEROL METHYLTRANSFERASE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application Serial No. 60/053,819 filed July 25, 1997 and U.S. Provisional Application Serial No. 60/072,497 filed January 26, 1998.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

Vitamin E is an essential component of mammalian diets. Epidemiological evidence indicates that Vitamin E supplementation results in decreased risk for cardiovascular disease and cancer, aids in immune function, and generally prevents or slows a number of degenerative disease processes in humans (Traber and Sies, Annu. Rev. Nutr. 16:321-347, 1996). Vitamin E functions in stabilizing the lipid bilayer of biological membranes (Skrypin and Kagan, Biochim. Biophys. Acta 815:209 1995; Kagan, N.Y. Acad. Sci. p 121, 1989; Gomez-Fernandez et al., Ann. N.Y. Acad. Sci. p 109, 1989), reducing polyunsaturated fatty acid (PUFA) free radicals generated by lipid oxidation (Fukuzawa et al., Lipids 17: 511-513, 1982), and quenching singlet oxygen species (Fryer, Plant Cell Environ. 15(4):381-392, 1992).

Vitamin E, or α-tocopherol, belongs to a class of lipid-soluble antioxidants that includes α, β, γ, and δ-tocopherols and α, β, γ, and δ-tocotrienols. Although α, β, γ, and δ-tocopherols and α, β, γ, and δ-tocotrienols are sometimes referred to collectively as "Vitamin E" in the popular press,

Vitamin E is properly defined chemically solely as α-tocopherol. Of the various tocopherols present in foodstuff, α-tocopherol is the most significant for human health both because it is the most bioactive of the tocopherols and also because it is the tocopherol most readily absorbed and retained by the body (Traber and Sies, Annu. Rev. Nutr. 16:321-347, 1996). The *in vivo* antioxidant activity of α-tocopherol is higher than the antioxidant activities of β, γ, and δ-tocopherol (Kamal-Eldin and Appelqzvist Lipids 31:671-701, 1996).

Only plants and certain other photosynthetic organisms, including cyanobacteria, synthesize tocopherols. Therefore, dietary tocopherols are obtained almost exclusively from plants. Plant tissues vary considerably in total tocopherol content and tocopherol composition. The predominant tocopherol in green, photosynthetic plant tissues often is α-tocopherol. Leaf tissue can contain from 10-50 μg total tocopherols/gram fresh weight.

Non-green plant tissues and organs exhibit a wider range of both total tocopherol levels and tocopherol compositions. In general, most of the major food staple corps (e.g., rice, corn, wheat, potato) produce low to extremely low levels of total tocopherols, of which only a small percentage is α-tocopherol (Hess, Vitamin E, α-tocopherol, *In* Antioxidants in Higher Plants, R. Alscher and J. Hess, Eds. 1993, CRC Press, Boca Raton. pp 111-134). Oil seed crops generally contain much higher levels of total tocopherols; however, α-tocopherol is present only as a minor component and β, γ, and δ-tocopherols and tocotrienols predominate (Taylor and Barnes, Chemy Ind., Oct.:722-726, 1981).

Daily dietary intake of 15-30 mg of vitamin E is recommended to obtain optimal plasma α-tocopherol levels. It is quite difficult to achieve this level of vitamin E intake

from the average American diet. For example, one could obtain the recommended daily dose of Vitamin E by daily consumption of over 750 grams of spinach leaves (in which α-tocopherol comprises 60% of total tocopherols) or 200-400 grams of soybean oil.

One alternative to relying on diet alone to obtain the recommended levels of vitamin E is to take a vitamin E supplement. However, most vitamin E supplements are synthetic vitamin E having six stereoisomers, whereas natural vitamin E vitamin is a single isomer. Furthermore, supplements tend to be relatively expensive, and the general population is disinclined to take vitamin supplements on a regular basis.

Although tocopherol function in plants has been less extensively studied than tocopherol function in mammalian systems, it is likely that the analogous functions performed by tocopherols in animals also occur in plants. In general, plant tocopherol levels have been found to increase with increases in various stresses, especially oxidative stress. Increased α-tocopherol levels in crops are associated with enhanced stability and extended shelf life of fresh and processed plant products (Peterson, Cereal-Chem 72(1):21-24, 1995; Ball, Fat-soluble vitamin assays in food analysis. A comprehensive review. London: Elsevier Science Publishers LTD, 1988).

Vitamin E supplementation of swine, beef, and poultry feeds has been shown to significantly increase meat quality and extend the shelf life of post-processed meat products by retarding post-processing lipid oxidation, which contributes to the formation of undesirable flavor components (Ball, *supra* 1988; Sante and Lacourt, J. Sci. Food Agric. 65(4):503-507, 1994; Buckley et al., J. of Animal Science 73:3122-3130, 1995).

What would be useful for the art is a method to increase the ratio of α-tocopherol to γ-tocopherol in seeds, oils, and leaves from crop and forage plants, or a method for producing natural vitamin E in nonphotosynthetic bacteria or fungi using

a large scale fermentation process. Increasing α-tocopherol levels in crop plants would increase the amount of α-tocopherol obtained in the human diet, and would enhance the stability and shelf life of plants and plant products. The meat industry would benefit from the development of forage plants having increased levels of vitamin E.

BRIEF SUMMARY OF THE INVENTION

The present invention is based on an isolated DNA fragment including a coding sequence for a γ-tocopherol methyltransferase.

The invention is also a heterologous genetic construct comprising a γ-tocopherol methyltransferase coding sequence operably connected to a plant, bacterial, or fungal promoter not natively associated with the γ-tocopherol methyltransferase coding sequence.

Another aspect of the present invention is a method of altering the tocopherol profile of a plant comprising the steps of: (a) providing a heterologous genetic construct comprising a γ-tocopherol methyltransferase coding sequence operably connected to a plant promoter not natively associated with the coding sequence; and (b) introducing the construct into the genome of a plant.

The present invention is also directed toward transgenic plants which have an altered ratio of α-tocopherol to γ-tocopherol, thus increasing the nutritive value of the plants and products therefrom for human and animals.

In another embodiment, the invention is a plant comprising in its genome a heterologous genetic construct comprising a γ-tocopherol methyltransferase coding sequence operably connected to a promoter that is functional in plants.

It is an object of the present invention to provide a genetic construct comprising a coding sequence for a γ-tocopherol methyltransferase operably connected to a plant

promoter not natively associated with the coding sequence which when expressed in a plant comprising the construct in its genome results in an alteration in the ratio of α-tocopherol:γ-tocopherol in the plant, relative to an untransformed wild-type plant.

It is an object of this invention to provide a plant having an altered α-tocopherol:γ-tocopherol ratio.

Other objects, features, and advantages of the invention will become apparent upon review of the specification and claims.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1 shows the alignment of amino acid sequences of γ-tocopherol methyl-transferases from *Arabidopsis thaliana* and *Synechocystis*. Inverted triangles denote putative cleavage sites of N-terminal targeting domains; the closed circle denotes the position of an in-frame *Nco*I site in the leader peptide of SLR0089.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is, in part, directed to a plant comprising in its genome a genetic construct comprising a γ-tocopherol methyltransferase coding sequence operably connected to a plant promoter not natively associated with the coding sequence. Such transgenic plants exhibit an altered ratio relative to the wild type plants of the same species. In fact, seed and seed oil of a plant not normally containing α-tocopherol can be altered so that the most abundant tocopherol is α-tocopherol. Alternatively, the relative percentage of γ-tocopherol present in plant tissue may be increased by reducing the activity of γ-tocopherol methyltransferase in the plant, which could be accomplished by expression of a γ-tocopherol

methyltransferase coding sequence in the antisense orientation. The development of plants with increased γ-tocopherol may be useful in certain industries.

Tocopherols and plastoquinones, the most abundant quinones in plant plastids, are synthesized by a common pathway (Hess, Antioxidants in Higher Plants, CRC Press: Boca Raton p 140-152, 1993; Soll, Plant Cell Membranes, Academic Press: San Diego p 383-392, 1987). The synthesis of tocopherols involves four steps catalyzed by at least six enzymatic activities. A branchpoint in the common pathway occurs upon phytylation or prenylation of the precursor homogentisic acid to form either 2-methyl-6-phytylplastoquinol or 2-methyl-6-solanylplastoquinol, intermediates in tocopherol and plastoquinone biosynthesis, respectively.

The intermediate 2-methyl-6-phytylplastoquinol is the common precursor to the biosynthesis of all tocopherols. In spinach leaves, the intermediate undergoes ring methylation to yield 2,3-dimethyl-6-phytylplastoquinol, which is cyclized to form γ-tocopherol. A second ring methylation at position 5 yields α-tocopherol (Soll and Schultz, Phytochemistry 19(2):215-218, 1980). The second ring methylation is catalyzed by γ-tocopherol methyltransferase, a distinct enzymatic activity from the methyltransferase that catalyzes the methylation at position 7, and the only enzyme of the pathway that has been purified from plants (d'Harlingue and Camara, J. Biol. Chem. 260(68): 15200-15203, 1985; Ishiko et al., Phytochemistry 31(5):1499-1500, 1992).

The methylation enzymes are involved in regulating the final composition of the tocopherol pool. Data obtained in studies of sunflower mutants suggest that the enzymes involved in methylation have a high degree of influence over relative tocopherol amounts but do not affect the overall regulation of total tocopherol content(Demurin, Helia 16:59-62, 1993). Normally, seed tocopherol composition in cultivated sunflower

(*Helianthus annuus* L.) is primarily α-tocopherol (i.e., 95-100% of the total tocopherol pool) (Skoric et al., Proceedings of the 14th International Sunflower Conference. 1996. Beijing/Shenyang, China). However, two mutant sunflower lines were identified with tocopherol compositions of 95% γ-tocopherol/5% α-tocopherol and 50% β-tocopherol/50% α-tocopherol. Although these presumed tocopherol methylation mutants were found to have dramatically different tocopherol profiles in seed, total tocopherol levels were not significantly different than those of wild type sunflower (Demurin, *supra* 1993). Based on these results, we hypothesized that it should be possible to alter the tocopherol profile of many plant species by manipulating γ-tocopherol methyltransferase expression without affecting the total tocopherol pool size.

The enzyme γ-tocopherol methyltransferase catalyzes the methylation of γ-tocopherol to form α-tocopherol, the final step in α-tocopherol biosynthesis. Overexpression of a γ-tocopherol methyltransferase gene in a plant enhanced the conversion of γ-tocopherol to α-tocopherol in any tissue containing γ-tocopherol, thereby increasing the α-tocopherol:γ-tocopherol ratio. In fact, seed and oil in which little or no α-tocopherol is found can be altered to contain predominantly α-tocopherol. Conversely, expression of the antisense RNA would be expected to reduce expression of the γ-tocopherol methyltransferase, causing a decrease in the α-tocopherol:γ-tocopherol ratio. Plants having increased γ-tocopherol may be useful for certain industries.

We have discovered that γ-tocopherol methyltransferase also catalyzes the conversion of δ-tocopherol to β-tocopherol. Overexpression of γ-tocopherol methyltransferase in plant tissue results in increased conversion of δ-tocopherol to β-

tocopherol. It is expected that expression of γ-tocopherol methyltransferase antisense RNA would result in reduced conversion of δ-tocopherol to β-tocopherol.

As demonstrated in the examples below, the seed of *Arabidopsis* plants transformed with a genetic construct comprising an Arabidopsis γ-tocopherol methyltransferase gene under the control of either the seed specific promoter or the constitutive cauliflower mosaic virus 35S promoter exhibit a dramatic increase in the ratio of α-tocopherol:γ-tocopherol. No α-tocopherol is detected in the seed of untransformed *Arabidopsis,* whereas seed from *Arabidopsis* transformed with the γ-tocopherol methyltransferase gene under the control of the seed-specific promoter contained about 90% α-tocopherol. Seed from *Arabidopsis* transformed with the γ-tocopherol methyltransferase gene under the control of a constitutive promoter contained slightly less α-tocopherol (84%). This observation demonstrates that for plants natively having a tocopherol profile in which α-tocopherol is not predominant (i.e. is less than 50% of total tocopherol), that α-tocopherol can be made to be the predominant tocopherol form in seed or seed oil from a transgenic plant.

Methylation of γ-tocopherol to form α-tocopherol is the means by which the ratio of the di-methylated tocopherols (γ-tocopherol) and tri-methylated tocopherol (α-tocopherol) is regulated. By up regulating γ-tocopherol methyltransferase expression in tissues in which it is not normally expressed in a plant, it is now possible to increase α-tocopherol levels in tissues of many agricultural crops in which γ-tocopherol is a major tocopherol (e.g., maize, soybean, rapeseed, cotton, peanut, safflower, castor bean, rice). Many common edible seed oils have large amounts of γ-tocopherol. Increasing the level of expression of γ-tocopherol methyltransferase in seed oil

plants should increase the ratio of α-tocopherol:γ-tocopherol.

Isolation and functional analysis of the γ-tocopherol methyltransferase genes from *Synechocystis* PCC6803 and *Arabidopsis thaliana* was accomplished by concurrently pursuing the complementary molecular genetic approaches described in detail in the examples. These two model organisms were selected because both synthesize tocopherols by similar or identical pathways and both are highly tractable genetic, molecular, and biochemical systems.

The DNA sequences of the γ-tocopherol methyltransferase genes from *Synechocystis* PCC6803 and *Arabidopsis thaliana* are shown in SEQ ID NO:1 and SEQ ID NO:3, respectively. The corresponding deduced amino acid sequences of the proteins are shown in SEQ ID NO: 2 and SEQ ID NO:4.

It is expected that the present invention may be practiced using a γ-tocopherol methyltransferase gene from any photosynthetic organism. It is well within the ability of one of skill in the art to isolate a plant γ-tocopherol methyltransferase gene using the sequences disclosed herein. The usefulness of these sequences to identify other γ-tocopherol methyltransferase coding sequences is demonstrated by the fact that it was the *Synechocystis* sequence that was used to identify the Arabidopsis sequence. The two sequences can be used to screen public computer databases of plant cDNAs (dbest databases) and genomic sequences. Alternatively, the sequences could be used to design probes for use in identifying genomic or cDNA clones containing a γ-tocopherol methyltransferase sequence. Another approach would be to use the sequences to design oligonucleotide primers for use in PCR amplification of γ-tocopherol methyltransferase genes from plant DNA.

To determine whether one has identified a γ-tocopherol methyltransferase sequence, one could perform a gene

replacement study using wild type *Synechocystis*, a complementation study using a *Synechocystis* γ-TMT knockout mutant, or an *in vitro* enzyme assay using a suitable substrate and γ-tocopherol methyltransferase protein expressed in *E. coli* or another suitable expression system. A genetic construct comprising the γ-tocopherol methyltransferase coding sequence operably connected to a plant promoter can be constructed and used to transform *Arabidopsis* or a plant or crop plant of interest. A transgenic plant comprising the construct in its genome would be expected to have altered expression of γ-tocopherol methyltransferase and an altered tocopherol profile relative to an untransformed, wild-type plant.

It is expected that polyploid plants having more than one copy of the γ-tocopherol methyltransferase gene may have allelic variations among γ-tocopherol methyltransferase gene sequences. It is anticipated that putative γ-tocopherol methyltransferase gene sequences having less than 100% homology to SEQ ID NO:1 or SEQ ID NO:3 encode proteins having γ-tocopherol methyltransferase activity.

It is envisioned that minor sequence variations from SEQ NO:1 or SEQ ID NO:3 associated with nucleotide additions, deletions, and mutations, whether naturally occurring or introduced *in vitro*, will not affect γ-tocopherol methyltransferase activity. The scope of the present invention is intended to encompass minor variations in γ-tocopherol methyltransferase sequences. Also, it is now well within the level of ordinary skill in the art of plant genetic engineering to alter the coding sequence for a gene by changing codons specifying for common amino acids or by making conservative amino acid substitutions at DNA sequences encoding non-critical portions of enzymes.

Construction of an expression vector comprising a γ-tocopherol methyltransferase coding sequence operably connected

to a plant promoter not natively associated with the coding sequence will be achieved using standard molecular biology techniques known to the art. The plant promoter may be a tissue-specific promoter such as a seed-specific promoter (e.g., napin or DC3), a constitutive promoter such as CaMV 35S, a developmental stage-specific promoter, or an inducible promoter. Promoters may also contain certain enhancer sequence elements that improve efficiency of transcription. Optionally, the construct may contain a termination signal, such as the nopaline synthase terminator (NOS). Preferably, the constructs will include a selectable or screenable marker to facilitate identification of transformants. The constructs may have the coding region in the sense or antisense orientation.

Once a genetic construct comprising a γ-tocopherol methyltransferase gene has been obtained, it can readily be introduced into a plant or plant tissue using standard methods known to the art. For example, the *Agrobacterium* transformation system is known to work well with all dicot plants and some monocots. Other methods of transformation equally useful in dicots and monocots may also be used. Transgenic plants may be obtained by particle bombardment, electroporation, or by any other method of transformation known to one skilled in the art of plant molecular biology. The experience to date in the technology of plant genetic engineering has taught that the method of gene introduction does not affect the phenotype achieved in the transgenic plants.

A transgenic plant may be obtained directly by transformation of a plant cell in culture, followed by regeneration of a plant. More practically, transgenic plants may be obtained from transgenic seeds set by parental transgenic plants. Transgenic plants pass on inserted genes, sometimes referred to as transgenes, to their progeny by normal Mendelian inheritance just as they do their native genes. Methods for breeding and regenerating plants of agronomic

interest are known to the art. Experience with transgenic plants has also demonstrated that the inserted gene, or transgene, can be readily transferred by conventional plant breeding techniques into any desired genetic background.

It is reasonable to expect that the expression of heterologous γ-tocopherol methyltransferase in a transgenic plant will result in alterations in the tocopherol profile in that plant. In addition to the inherent advantage of increasing the α-tocopherol:γ-tocopherol ratio, changes in the tocopherol profile may result in unique, advantageous phenotypes. This invention is intended to encompass other advantageous phenotypes that may result from alterations in tocopherol biosynthesis in plants obtained by the practice of this invention.

Using the information disclosed in this application and standard methods known to the art, one of skill in the art could practice this invention using any crop plant or forage plant of interest.

The following nonlimiting examples are intended to be purely illustrative.

EXAMPLES

Example 1. Identification and Characterization of a Putative γ-TMT Gene in *Synechocystis* PCC6803

We recently cloned and characterized the γ-tocopherol methyltransferase gene from *Synechocystis* as follows. An *Arabidopsis* p-hydroxyphenyl-pyruvic acid dehydrogenase (HPPDase) cDNA sequence (Norris and Della Penna, submitted, Genbank Accession # AF000228, Plant Physiol., in press) was used to search a database containing the DNA sequence of the *Synechocystis* PCC6803 genome (Kaneko et al., DNA Res. 3:109-136, 1996). We identified an open reading (designated SLR0090) that shares a high degree of amino acid sequence similarity (i.e. 35% identity and 61% similarity) with the *Arabidopsis*

HPPDase enzyme. The putative *Synechocystis* HPPDase gene is located within an operon in the *Synechocystis* genome comprised of 10 open reading frames (ORFs) encompassing bases 2,893,184 to 2,905,235 of the published *Synechocystis* PCC6803 genome (Kaneko et al., *supra* 1996). We hypothesized that this operon might also contain additional genes that encode other enzymes involved in tocopherol synthesis.

Two ORFs (SLR0089 and SLR0095) were identified as possible candidates for *Synechocystis* tocopherol methyltransferase genes. BLAST searches with ORFs SLR0089 and SLR0095 showed that these proteins share a high degree of similarity to the known protein sequences of Δ-(24)-sterol-C-methyltransferases and various plant caffeol CoA-O-methyltransferases, respectively. Both SLR0089 and SLR0095 proteins contain consensus sequences corresponding to conserved S-adenosyl-methionine (SAM) binding domains (Kagan and Clarke, Archives of Biochem. and Biophy. 310(2):417-427, 1996). The SLR0089 protein contains other structural features that are consistent with features found in a tocopherol methyltransferase. These features were not found in SLR0095. First, PSORT (Prediction of Protein Localization Sites) computer analysis of the two protein sequences predict that SLR0089 is localized to the plasma membrane, whereas and SLR0095 is localized to the cytosol. Tocopherol biosynthesis in cyanobacteria is believed to occur in the plasma membrane; therefore, localization of SLR0089 protein to the plasma membrane suggests that it may be a tocopherol methyltransferase. Additionally, PSORT analysis identified the presence of a putative bacterial signal sequence in the first 25 amino acids of the SLR0089 protein. The predicted molecular weight of the mature SLR0089 protein (after truncation of the signal sequence) is 32,766 daltons, which is very close to the reported molecular weight (33,000 daltons) of purified from pepper fruits (d'Harlingue and Camara, *supra* 1985). The predicted molecular weight of SLR0095 is 24,322

daltons. Therefore, we concluded that of the two identified ORFs, the SLR0089 gene was more likely to be a tocopherol methyltransferase.

Example 2. Amplification and cloning of the *Synechocystis* γ-TMT gene

Synechocystis genomic DNA was isolated by the method of Williams (Methods Enzymol.167:776-778, 1987). The SLR0089 gene was amplified from *Synechocystis* genomic DNA by polymerase chain reaction (PCR) using a sense strand specific primer(SLR0089F, SEQ ID NO:5) and a non-sense strand specific primer SLR0089R (SEQ ID NO:6) under the following conditions:

The amplification of the SLR0089 open reading frame was conducted in a 50μl reaction volume containing 0.4 mM dATP, 0.4 mM dGTP, 0.4 mM dCTP, 0.4 mM dCTP, 0.4 mM dTTP, 0.2 μM SLR0089F primer, 0.2 μM SLR0089R primer, 10 ng Synechocystis PCC6803 genomic DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, and 2.5 units *Taq* polymerase (Gibco-BRL). PCR thermocycle conditions were performed as follows:

5 minutes 95°C (1 cycle)
1 minute 95°C -> 1 minute 55°C -> 1.5 minutes 72°C (35 cycles)
7 minutes 72°C (1 cycle)

The PCR product comprising the SLR0089 ORF was cloned using standard molecular biological techniques known to one of skill in the art. Briefly, the amplified SLR0089 ORF was purified and made blunt ended by treatment with the Klenow fragment. The SLR0089 gene was ligated to *Eco*RV-linearized pBluescript KS II (Stratagene, Inc., LaJolla, CA). The ligation mixture was used to transform competent *E. coli* DH5α cells, and putative transformants were selected on the basis of ampicillin resistance. A plasmid designated pH-1 that was isolated from a transformant was found to contain the SLR0089

insert. The identity of the SLR0089 gene (SEQ ID NO:1) was confirmed by sequencing using T7 and T3 sequencing primers.

Example 3. Development of a SLR0089 knockout mutant

A gene replacement vector was constructed using standard molecular biology techniques. The plasmid pH1, which contains a unique NcoI site in the SLR0089 ORF, was digested with NcoI restriction endonuclease. The aminoglycoside 3'-phospho-transferase gene from Tn903 was ligated to the *Nco*I site of pH1 and the ligation mixture was used to transform *E. coli* DH5α cells. Transformants were selected using kanamycin and ampicillin. A recombinant plasmid (pQ-1) containing the disrupted SLR0089 ORF was isolated and used to transform Synechocystis PCC6803 according to the method of Williams (Methods Enzymol.167:776-778, 1987).

Synechocystis transformants were selected for on BG-11 medium (Castenholz, Methods in Enzymology p 68-93, 1988) containing 15 mM glucose and 15 μg/ml kanamycin. All cultures were grown under continuous light at 26°C. Four independent transformants were carried through five subculturings of single colonies to fresh medium. PCR and genomic analysis were used to confirm that the gene replacement was successful and complete.

Example 4. Tocopherol profiles of wild type and mutant Synechocystis

Approximately 200 mg of cells were scraped from 2 week old *Synechocystis* cultures grown on BG-11 agar medium. The cells were homogenized in 6 ml of 2:1 (volume:volume) methanol:$CHCl_3$ containing 1 mg/ml butylated hydroxytolulene (BHT) using a polytron homogenizer. Following homogenization, 2 ml of $CHCl_3$ and 3.4 ml of double-distilled water was added to the homogenate. The lower lipid phase was removed and dried under nitrogen gas. The dried lipids were resuspended in 200μl of

HPLC grade ethyl acetate containing 1 mg/ml BHT.

Tocopherols were analyzed by reverse phase HPLC using a Hewlett-Packard Series 1100 HPLC system with a fluorescence detector. Crude lipid extracts were fractionated on a Water Spherisorb S5 ODS2 4.6 X 250 mm column in a mobile phase consisting of 75% methanol and 25% isopropanol and a flow rate of 1 ml/min. The fluorescence was measured at 330 nm after excitation at a wavelength of 290 nm.

Wild-type *Synechocystis* produces α-tocopherol as its most abundant tocopherol (>95%of total tocopherols). The SLR0089 disrupted mutant of *Synechocystis* is no longer able to synthesize α-tocopherol and instead accumulates γ-tocopherol as its sole tocopherol. The elimination of α-tocopherol production and concomitant accumulation of γ-tocopherol conclusively demonstrates that SLR0089 encodes γ-tocopherol methyltransferase, the final step in α-tocopherol biosynthesis.

Example 5. Identification of a Putative *Arabidopsis* γ-TMT cDNA from the EST Database

The *Arabidopsis* EST database (Ausbel et al., Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, N.Y., 1987) was searched using the *Synechocystis* γ-TMT DNA and protein sequences as queries. Two cDNA clones that share significant homology with the *Synechocystis* sequence were identified: the *Arabidopsis* Δ-(24)-sterol-C-methyltransferase and the *Arabidopsis* expressed sequence tag (EST) clone 165H5T7. Because the Δ-(24)-sterol-C-methyltransferase was functionally identified by its ability to complement a yeast Δ-(24)-sterol-C-methyltransferase mutant (*erg6*), we are confident that the clone does not encode a γ-TMT (Husselstein *et al.*, FEBS Letters 381:87-92, 1996). Therefore, we decided to focus our efforts on the *Arabidopsis* 165H5T7 EST clone (Genbank Accession #R30539). The DNA sequence of the

165H5T7 EST clone was determined (SEQ ID NO:3) and the amino acid sequence of the putative protein was deduced. The sequence was aligned with that of the *Synechocystis* γ-TMT (Fig 1). The full-length 165HT7 clone encodes a protein that is 35% identical and 66% similar to the Synechocystis γ-TMT and exhibits large blocks of identity. When 165H5T7 was used as query against the non-repetitive protein database, it was found to have the highest homology to SLR0089 ($P<10^{-54}$) and only moderate homology to the four known plant Δ-(24)-sterol-C-methyltransferases ($P\geq10^{-5}$). 165H5T7 also contains conserved SAM binding motifs common to a large number of methyltransferases (Fig. 1) but lacks proposed sterol binding domains common in the four plant Δ-(24)-sterol-C-methyltransferases identified to date (Husselstein *et al.*, *supra* 1990). These data suggest that clone 165H5T7 encodes an *Arabidopsis* γ-TMT homologue, which we have designated A.t.γ-TMT.

Example 6. Characterization of the putative *Arabidopsis* γ-TMT homologue using the gene replacement in *Synechocystis*

Plant cDNAs encoding putative γ-TMT homologues may be functionally identified using one of two gene replacement approaches in *Synechocystis*. One approach that may be employed is to replace the endogenous *Synechocystis* γ-TMT gene in wild type *Synechocystis* with the putative Arabidopsis γ-TMT cDNA 165H7T7. A *Synechocystis* γ-TMT(coding sequence # SLR0089) gene replacement vector will be constructed to include the following features, in 5' to 3' order: 1) at least 300 base pairs of DNA sequence corresponding to the *Synechocystis* genomic sequence found immediately upstream (5') of the native SLR0089 gene; 2) the first 77 base pairs of the SLR0089 ORF corresponding to the identified bacterial signal sequence that ends with a unique,

in-frame *Nco*I site; 3) a polylinker or multiple cloning site; 4) an antibiotic resistance marker (e.g., a kanamycin resistance gene cassette); and 5) at least 300 base pairs of DNA sequence corresponding to the *Synechocystis* genomic sequence found immediately downstream (3') of the native SLR0089 gene. The putative plant γ-TMT cDNA to be tested for complementation will be inserted into the NcoI site or into the multiple cloning site.

The 165H5T7 cDNA may be engineered to contain an *Nco*I site at the transit peptide cleavage site predicted by PSORT using PCR mutagenesis, which would change the amino acid Val-48 to Met. The cDNA owill be ligated to the unique *Nco*I site in the SLR0089 gene replacement plasmid to create an in-frame, amino-terminal fusion between the *Synechocystis* γ-TMT signal peptide and the plant protein sequence. The construct will be used to transform wild type *Synechocystis;* transformants will be identified by kanamycin selection. After several single colony passages under selection, gene replacement will be confirmed by PCR. The tocopherol profile of transformants will be determined by HPLC. *Synechocystis* transformants functionally expressing *Arabidopsis* γ-TMT genes will be identified by their ability to synthesize α-tocopherol in the absence of a functional *Synechocystis* γ-TMT gene.

In an alternative approach, the putative γ-TMT gene may be characterized according to its ability to complement the *Synechocystis* γ-TMT knockout mutant. The replacement vector could be constructed to include the intact putative γ-TMT gene and an antibiotic resistance marker other than kanamycin. Following transformation and selection, gene replacement can be confirmed by PCR and the transformants may be further characterized by tocopherol analysis.

Example 7. Functional characterization of *Arabidopsis* and *Synechocystis* γ-TMT genes by expression in *E. coli*

The proteins encoded by the *Synechocystis* SLR0089 gene and the *Arabidopsis* 165h5T7 cDNA clone were identified as γ-TMTs through functional expression in E. coli.

The SLR0089 gene was amplified from the *Synechocystis* PCC6803 genome using polymerase chain reaction (PCR). The forward primer (SLR0089coliF, SEQ ID NO:7), was designed to add a *Bsp*HI site to the 5' end of the primer. The reverse (3') PCR primer (SLR0089coliR, SEQ ID NO:8) was designed with a *Bgl*II site engineered at the 5' end of the primer.

The PCR reaction was conducted in two 100-μl reaction mixtures, each of which contained dNTPs (0.4 mM each), 2 μM SLR0089coliF, 2 μM SLR0089coliR, 10 ng *Synechocystis* PCC6803 genomic DNA, 10 mM KCl, 6.0 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.2), 2 mM $MgCl_2$, 0.1% Triton X-100, 10 μg/ml BSA, 2.5 units Pfu polymerase (Stratagene, LaJolla, CA). The following thermocycle conditions were used:

5 minutes 95°C (1 cycle)
0.75 minutes 94°C -> 0.75 minutes 55°C -> 2 minutes 72°C (30 cycles)
10 minutes 72°C (1 cycle)

The PCR fragment was gel-purified and ligated to *Eco*RV-linearized pBluescript KS II (Stratagene, LaJolla, CA). The ligation product was used to transform *E. coli* strain DH5α, and putative transformants were selected on the basis of ampicillin resistance. A recombinant plasmid containing the insert (designated p082297) was sequenced to confirm the correct amplification and subcloning of the SLR0089 sequence.

The deduced amino acid sequence of SLR0089 contains a putative amino-terminal bacterial signal sequence comprising the first 24 amino acids of the deduced amino acid sequence. Because this amino-terminal signal sequence could effect the conformation of the SLR0089 protein when expressed in *E. coli* and render the protein inactive, we modified the SLR0089 DNA sequence such that it encodes a truncated protein devoid of the putative amino-terminal bacterial signal sequence. The SLR0089 gene contains a *Nco*I recognition sequence at the predicted cleavage site for the putative bacterial signal sequence. A *Nco*I-*Bgl*II fragment containing a truncated SLR0089 DNA sequence from p082297-coli was subcloned in the correct reading frame into the *Nco*I and *Bam*HI sites of the T7 *E. coli* pET3D expression vector (Novagen, Madison, WI). The ligation mixture was used to transform *E. coli* BL21 (DE3) and transformants were selected for on the basis of ampicillin resistance. A plasmid (designated p011698-1) containing the insert was identified by restriction digest analysis with the enzyme *Hind*III.

The 165H5T7 cDNA clone was also subcloned into the pET3D expression vector. The first 50 N-terminal amino acids of the deduced amino acid sequence of 165H5T7 contains a putative amino-terminal chloroplast targeting sequence that could effect the conformation of the 165H5T7 protein when expressed in E. coli and render the protein inactive. Therefore, we modified the 165H5T7 DNA sequence to encode a truncated protein devoid of the putative amino-terminal chloroplast targeting sequence. The truncated 165H5T7 DNA sequence was obtained by PCR amplification of 165H5T7 cDNA using primers designed to amplify the sequence corresponding to the region between nucleotide 353 and nucleotide 1790 of the original 165H5T7 sequence. The forward PCR primer (165matF, SEQ ID NO:9) adds a *Nco*I site to the 5' end of the truncated 165H5T7 sequence to facilitate cloning into the pET3D vector. The reverse (3') PCR primer (165matR, SEQ ID NO:10) was designed from the polylinker region

of the pSPORT1 vector with a *Acc*I site engineered at the 5' end of the primer. The PCR reaction was conducted with the 165matF and 165matR primers (2μM each) using the same PCR conditions described for the amplification of the truncated Synechocystis gene, above.

Following gel purification, the PCR fragment was ligated to EcoRV-linearized pBluescript KS II, the ligation product was used to transform *E. coli* strain DH5α, and ampicillin-resistant putative transformants were selected. A recombinant plasmid (designated p010498-2) containing the insert was identified. The DNA sequence of p010498-2 was determined to confirm the correct amplification and subcloning of the truncated 165H5T7 sequence. The truncated 165H5T7 DNA sequence was subcloned as a *Nco*I-*Bam*HI fragment pET3D vector digested with *Nco*1 and *Bam*HI. The ligation product was used to transformed *E. coli* DH5α and transformants were selected for on the basis of ampicillin resistance. A plasmid (designated p011898-1) containing the insert was identified by restriction digest analysis with the enzyme *Hind*III.

The p011698-1 and p011898-1 constructs were used to transform the *E. coli* T7 expression host BL21(DE3). To generate protein for γ-TMT assays, one liter cultures of transformed host cells containing one of the constructs were grown in Luria broth containing 100 mg/liter ampicillin. Each culture was started at an optical density at 600 nm (OD_{600}) of 0.1 and incubated in a shaking incubator at 28°C until the culture reached an OD_{600} of 0.6, at which time isopropyl-β-D-thiogalactopyranoside (IPTG) was added to each culture to obtain a final concentration of 0.4 mM IPTG. Each culture was incubated for an additional 3 hours at 28°C and the cells were harvested by centrifugation at 8,000 g. The cell pellets were then resuspended in 10 ml of 10 mM HEPES (pH 7.8), 5 mM DTT, 0.24 M sorbitol, 1 mM PMSF. The cells were lysed by sonication with a micro-tip sonicator using four 10-second pulses. Triton

X 100 was added to each homogenate to a final concentration of 1%. The homogenates were incubated on ice for 30 minutes, and subjected to centrifugation at 30,000 g for 30 minutes at 4°C. The supernatants of these extracts were assayed for γ-tocopherol methyltransferase activity as follows.

The γ-TMT assays were performed in 250 μl volumes containing 50 mM Tris (pH 7.5 for the *Synechocystis* and pH 8.5 for the *Arabidopsis* enzyme), 5 mM DTT, 5 mM γ- or δ-tocopherol, and 0.025 μCi (55μCi/mmole) (^{14}C-methyl)-S-adenosylmethionine. Reaction mixtures were incubated at room temperature for 30 minutes. The reactions were stopped by adding of 1 ml of 2:1 (v:v) $CHCl_3$:methanol containing 1 mg/ml butylated hydroxytolulene (BHT) and 250 μl of 0.9% NaCl in water, and vortexing. The samples were centrifuged to separate the phases. The $CHCl_3$ (lower) phase was transferred to a fresh tube containing 100 mg of α-tocopherol and the $CHCl_3$ was then removed under vacuum in a speed-vac. The dried lipid fraction was resuspended in 50 μl ethyl acetate containing 1 mg/ml BHT. The lipid extracts were fractionated on silica 60 TLC plates in dichloromethane. Tocopherols were then identified by co-migration with authentic tocopherol standards after staining the plate with Emmerie-Engels solution (0.1% FeCl3, 0.25% 2,2'-dipyridyl in ethanol). The band corresponding to α-tocopherol was scraped from the TLC plate and the amount of radioactive material present was determined by scintillation counting. These experiments showed that the proteins encoded by the *Synechocystis* SLR0089 and *Arabidopsis* 165H5T7 DNA sequences were able to convert γ-tocopherol to α-tocopherol.

The *Synechocystis* and *Arabidopsis* γ-tocopherol methyltransferases were tested for activity using several different methyl-substituted tocopherol substrates. Both enzymes were able to specifically convert δ-tocopherol to β-tocopherol. The two enzymes were unable to use tocol, 5,7-

diemethyltocol, β-tocopherol, and γ-tocotrienol as substrates. These results indicate that both the *Synechocystis* and *Arabidopsis* γ-tocopherol methyltransferases catalyze the methylation of carbon 5 of the tocopherol chromanol ring. The *Synechocystis* and *Arabidopsis* γ-TMTs appear to require substrates with a methyl-group present on the 8 position of the chromanol ring and a fully saturated prenyl-tail for activity. Our results indicate that *Arabidopsis* γ-TMT exhibits greater activity with γ-tocopherol as the substrate than with the δ-tocopherol substrate, whereas the *Synechocystis* γ-TMT appears to be equally active toward γ-tocopherol and δ-tocopherol.

Example 8. Qualitative manipulation of tocopherols in Arabidopsis and other plants by over expressing the Arabidopsis γ-tocopherol methyltransferase.

The results from HPLC analysis of lipid extracts made from *Arabidopsis* leaves and seeds indicate that these tissues have relatively simple tocopherol profiles. In *Arabidopsis* leaves, α-tocopherol is present at ~90% of the total tocopherol content, with γ-tocopherol comprising the remainder of the tocopherol content. In *Arabidopsis* seeds, γ-tocopherol is present at ~95% of the total tocopherol content in Arabidopsis seeds with the remaining 5% being composed of δ-tocopherol. These simple tocopherol profiles make *Arabidopsis* seed and leaf tissue ideal targets for evaluating the functional consequences of altering the expression of a γ-tocopherol methyltransferase gene in plants.

We hypothesized that increasing the expression of a γ-tocopherol methyltransferase gene in *Arabidopsis* would increase α-tocopherol levels as a proportion of the total tocopherols. To test this hypothesis, the full-length *Arabidopsis* γ-tocopherol methyltransferase cDNA clone 165H5T7 was over-

expressed under the control of the strong constitutive cauliflower mosaic virus 35S transcript (CaMV 35S) promoter and the embryo-specific carrot DC3 promoter (Seffens WS *et al.*, Dev. Genet. 11: 65-76,1990) in transgenic *Arabidopsis*.

The seed-specific plant gene expression plasmid was constructed from a derivative of the *Agrobacterium* plant transformation vector, pBIB-Hyg (Becker, D. Nucleic Acids Res. 18:203, 1990). The carrot embryo DC3 promoter was isolated from the plasmid pBS-DC3 5' PH after digestion with *Hind*III and *Bam*HI. The DC3 *Hind*III and *Bam*HI promoter fragment was then treated with DNA polymerase to fill in the 5' over-hanging ends. The pBIB-Hyg plasmid was digested with *Hind*III and then treated with DNA polymerase to fill-in the 5' over-hanging ends. The DC3 promoter fragment was ligated to pBIB-Hyg to create a plasmid designated p111397. The *Arabidopsis* γ-tocopherol methyltransferase cDNA 165H5T7 was subcloned in the sense orientation as a *Sal*I-*Xba*I fragment into the *Sal*I and *Xba*I sites of p111397 to obtain p122997. The p122997 plasmid has the following features: 1) plant hygromycin selectable marker; 2) *Agrobacterium* T-DNA left and right border sequences; 3) the *Arabidopsis* 165H5T7 γ-tocopherol methyltransferase cDNA cloned between the carrot seed specific DC3 promoter and the nopoline synthase 3' transcriptional termination sequences; 4) the RK2 broad host bacterial plasmid origin of replication; and 5) bacterial kanamycin resistance selectable marker.

The constitutive *Arabidopsis* γ-tocopherol methyltransferase gene expression plasmid was derived from pSN506 CaMV 35S binary plant expression vector, a pART27 derivative in which the p-hydroxyphenol pyruvic acid dioxygenase (HPPDase) cDNA is under the control of the CaMV 35S promoter. (Norris and Della Penna, in press). The CaMV 35S/γ-tocopherol methyltransferase construct was made by replacing the HPPDase cDNA with the full length 165H5T7 cDNA sequence. The HPPDase cDNA fragment was removed from pSN506 by digesting

the plasmid with *Xba*I and *Xho*I. The 5' DNA over-hanging ends of the pSN506 *Xba*I-*Xho*I vector fragment were filled in using the Klenow fragment of the *E. coli* DNA polymerase. The linearized vector was ligated to a blunt-ended *Xba*I-*Sal*I fragment from 165H5T7 encoding the full length γ-tocopherol methyltransferase. A recombinant plasmid containing the insert was obtained and designated p010398. The plasmid p010398 contains the following characteristics: 1) plant kanamycin selectable marker; 2) agrobacterium T-DNA left and right border sequences; 3) the Arabidopsis 165H5T7 γ-tocopherol methyltransferase cDNA cloned between the CaMV 35S promoter and the nopoline synthase 3' transcriptional termination sequences; 4) the RK2 broad host bacterial plasmid origin of replication; and 5) bacterial kanamycin resistance selectable marker.

The constitutive and seed specific γ-tocopherol methyltransferase plant gene expression constructs (p122997 and p010398) and the appropriate empty vector control vectors (pART27 and p111397) were used to transform *Agrobacterium tumefaciens* strain C58 GV3101. Wild type *Arabidopsis* (ecotype Columbia) plants were transformed with these Agrobacterium strains using the vacuum infiltration method (Bechtold N, Ellis J, Pelletier G, in planta Agrobacterium mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. CR Acad Sci Paris, 1993. 1144(2): 204-212). Seeds from the primary transformants were selected for resistance to the appropriate antibiotic on medium containing MS salts, 1% sucrose, 0.7% agar, and suitable levels of the antibiotic. Antibiotic resistant seedlings (representing the T1 generation) were transferred to soil and grown to maturity. Leaf and seed material from these T1 generation plants were analyzed by HPLC.

Example 9. Characterization of Transgenic Plants.

A. Analysis of transgenic Arabidopsis Tocopherol Profiles

Known weights of approximately 5 mg of plant material (i.e. seed or leaf) and 100 ng of tocol (for use as an internal standard) were homogenized in 300 μl of 2:1 (V/V) methanol: $CHCl_3$ containing 1 mg/ml butylated hydroxytolulene (BHT). One hundred μl of $CHCl_3$ and 180 μl of 0.9% (w/v) NaCl in water were added to the homogenate and the mixture was briefly vortexed. The mixture was then centrifuged and the lower ($CHCl_3$) fraction was removed and transferred to a fresh tube. The $CHCl_3$ fraction was dried under vacuum and the resulting lipid residue was resuspended in 100 μl of ethyl acetate for analysis by C18 reverse phase HPLC or in 100 μl of hexane for analysis by normal phase HPLC.

Crude lipid extracts were analyzed by normal phase or reverse phase HPLC for changes in tocoperhol profiles. Individual tocopherol species were quantified by comparing their fluorescence signals with standard curves made from known quantities of authentic tocopherol standards. Reverse phase HPLC was done as describe in example 4. Normal phase HPLC analysis was done on a Licosorb Si60A 4.6 X 250 mm HPLC column using the following conditions:
Column temperature: 42°C

mobile phase: solvent A = HPLC grade hexane
solvent B = diisopropylether

Gradient : time	%solvent A	% solvent B	flow rate (ml/min)
0	92%	8%	1
20	82%	18%	1
25	82%	18%	1
25	92%	8%	2
34	92%	8%	2

Fluorescence Detector Settings:

excitation wavelength: 290 nm
emmission wavelentgh: 325nm

The concentrations of the various tocopherol species obtained by HPLC analysis of T1 seed material from *Arabidopsis* plants transformed with p122997, p010398, p111398, pART27 are shown in Table 1. Plants over-expressing the γ-tocopherol methyltransferase using either the CaMV 35S or carrot DC3 promoters are able to convert the majority of the γ-tocopherol normally present in *Arabidopsis* seeds to α-tocopherol and also are able to convert the majority of the δ-tocopherol normally present in *Arabidopsis* seeds to β-tocopherol. These results show that γ-tocopherol methyltransferase activity is normally limiting in *Arabidopsis* seeds.

B. Analysis of γ-tocopherol methyltransferase activity in transgenic Arabidopsis seed

Seeds from the T1 generation plants transformed with p122997, p010398, p111397, and pART27 were assayed for γ-tocopherol methyltransferase activity. Protein extracts were made by homogenizing approximately 10 mg of seeds in 200 μl of 50 mM Tris pH 8.5, 5 mM DTT, 1% Triton X 100, 1 mM PMSF. The extracts were centrifuged for 5 minutes to remove insoluble material. A 25-μl aliquot of each extract supernatant was assayed for γ-tocopherol methyltransferase activity as described in example 7. No γ-tocopherol methyltransferase activity was detected in wild type seeds and empty vector controls. Activity in seed-specific lines was approximately 2 pmol/hr/mg protein, and in 35S constitutive expression lines activity was 0.5 pmol/hr/mg protein.

Example 11. Other Transgenic Plants.

Based on this data demonstrating that a simple insert of a α-tocopherol methyl transferase gene into a plant can dramatically change the relative proportions of tocopherols in

a plant seed, it becomes possible to reasonably suggest the similar results that can be obtained in other plant species.

It is expected that one may manipulate tocopherol profiles in any plant species using the methods disclosed in the examples. Based on the concentration of the various tocopherols in untransformed plant tissue, we have predicted tocopherol profiles obtainable for a variety of plant tissue (Table 2). Note that several common plant oils (e.g. soybean) which are predominantly γ-tocopherol and contain low levels of α-tocopherol can be altered to be predominantly α-tocopherol.

All publications cited in this patent application are incorporated by reference herein.

The present invention is not limited to the exemplified embodiment, but is intended to encompass all such modifications and variations as come within the scope of the following claims.

Table 1

	ng α-tocopherol/ mg seed (% total tocopherol)	ng β-tocopherol/ mg seed (% total tocopherol)	ng γ-tocopherol/ mg seed (% total tocopherol)	ng δ-tocopherol/ mg seed (% total tocopherol)	ng total-tocopherol/mg seed (% total tocopherol)
122997-1 (seed specific promoter/ Arabidopsis γ-TMT)	523.28 ± 45.06 (88.91%)	23.91 ± 3.81 (4.06%)	41.38 ± 4.05 (7.03%)	ND (0%)	588.55 ± 48.02 (100%)
111397-2 (seed specific promoter /empty vector control)	ND (0%)	ND (0%)	409.16 ± 6.82 (95.11%)	17.81 ± 0.82 (4.89%)	430.19 ± 7.05 (100%)
010398-1 (constitutive promoter/ Arabidopsis γ-TMT)	373.85 ± 15.25 (83.74%)	17.16 ± 0.87 (3.84%)	55.41 ± 5.12 (12.41%)	ND (0%)	446.43 ± 18.46 (100%)
ART27-1 (constitutive promoter/empty vector control)	ND (0%)	ND (0%)	409.99 ± 7.00 (96.41%)	15.41 ± 0.11 (3.62%)	425.28 ± 6.80 (88.91%)

ND= none detected
All samples were analyzed in triplicate

Table 2

Crop Species (tissue)	Tocopherol composition of untransformed plant	Expected tocopherol composition of transgenic plants with γ-TMT over-expressed
Soybean[1] (seed/oil)	70% γ-tocopherol 22% δ-tocopherol 7% α-tocopherol 1% β-tocopherol	77% α-tocopherol 23% β-tocopherol
Oil Palm[1] (seed/oil)	25% α-tocopherol 30% α-tocotrienol 40% γ-tocotrienol 5% δ-tocotrienol	25% α-tocopherol 70% α-tocotrienol 5% β-tocotrienol
Peanut[2] (raw nut)	50% α-tocopherol 50% γ-tocopherol	100% α-tocopherol
Peanut[2] (nut oil)	33% α-tocopherol 66% γ-tocopherol	100% α-tocopherol
Safflower[2] (seed oil)	48% α-tocopherol 22% γ-tocopherol 30% δ-tocopherol	70% α-tocopherol 30% β-tocopherol
Rapeseed[2] (seed oil)	25% α-tocopherol 75% δ-tocopherol	100% α-tocopherol
Cotton Seed[1] (seed oil)	40% α-tocopherol 58% γ-tocopherol 2% δ-tocopherol	98% α-tocopherol 2% β-tocopherol
Wheat[2] (whole wheat flour)	20% α-tocopherol 7% α-tocotrienol 17% β-tocopherol 56% β-tocotrienol	20% α-tocopherol 7% α-tocotrienol 17% β-tocopherol 56% β-tocotrienol
Wheat[1] (germ oil)	75% α-tocopherol 25% γ-tocopherol	100% α-tocopherol
Corn[1] (oil)	22% α-tocopherol 68% γ-tocopherol 3% β-tocopherol 7% δ-tocopherol	90% α-tocopherol 10% β-tocopherol
Castor Bean[2] (oil)	50% γ-tocopherol 50% δ-tocopherol	50% α-tocopherol 50% β-tocopherol
Corn[2] (whole grain)	11% α-tocopherol 69% γ-tocopherol 4% α-tocotrienol 9% γ-tocotrienol 7% β-tocotrienol	80% α-tocopherol 13% α-tocotrienol 7% β-tocotrienol
Barley[2] (whole grain)	14% α-tocopherol	16% α-tocopherol

	2% γ-tocopherol 10% β-tocopherol 44% α-tocotrienol 7% γ-tocotrienol 23% β-tocotrienol	10% β-tocopherol 51% α-tocotrienol 23% β-tocotrienol
Rice[2] (whole grain)	50% α-tocopherol 50% γ-tocopherol	100% α-tocopherol
Potato[2] (tuber)	95% α-tocopherol 5% γ-tocopherol	100% α-tocopherol
Sunflower[2] (seeds raw)	95% α-tocopherol 5% γ-tocopherol	100% α-tocopherol
Sunflower[1] (seed oil)	96% α-tocopherol 2% γ-tocopherol 2% β-tocopherol	98% α-tocopherol 2% β-tocopherol
Banana[1] (fruit)	100% α-tocopherol	100% α-tocopherol
Lettuce[1] (leaf)	53% α-tocopherol 47% γ-tocopherol	100% α-tocopherol
Broccoli[2]	72% α-tocopherol 28% γ-tocopherol	100% α-tocopherol
Cauliflower[2]	44% α-tocopherol 66% γ-tocopherol	100% α-tocopherol
Cabbage[1]	100% α-tocopherol	100% α-tocopherol
Apple[2]	100% α-tocopherol	100% α-tocopherol
Pears[2]	93% α-tocopherol 7% γ-tocopherol	100% α-tocopherol
Carrots[2]	94% α-tocopherol 4% γ-tocopherol 2% δ-tocopherol	98% α-tocopherol 2% β-tocopherol

[1]McLaughlin, P.J, Weihrauch, J.C. "Vitamin E content of foods", J. Am. Diet Ass. 75:647-665 (1979).
[2]Bauernfeind, J. "Tocopherols in foods", In Vitamin E: A Comprehensive Treatise, L.J Machlin ed., Marcel Dekker, Inc. New York pp 99-168.